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Regulated expression of multiple chicken erythroid membrane skeletal protein 4.1 variants is governed by differential RNA processing and translational control

(erythropoiesis/membrane skeleton/multiple mRNAs)

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ABSTRACT **Protein 4.1 is an extrinsic membrane protein that facilitates the interaction of spectrin and actin in the erythroid membrane skeleton and exists as several structurally related polypeptides in chickens. The ratio of protein 4.1 variants is developmentally regulated during terminal differentiation of chicken erythroid and lenticular cells. To examine the mechanisms by which multiple chicken protein 4.1 variants are differentially expressed, we have isolated cDNA clones specific for chicken erythroid protein 4.1. We show that a single protein 4.1 gene gives rise to multiple 6.6-kilobase mRNAs by differential RNA processing. Furthermore, the ratios of protein 4.1 mRNAs change during chicken embryonic erythropoiesis. We observe a quantitative difference in variant ratios when protein 4.1 is synthesized *in vivo* or in a rabbit reticulocyte lysate *in vitro*. Our results show that the expression of multiple protein 4.1 polypeptides is regulated at the levels of translation and RNA processing.**

A network based on the proteins spectrin and actin lines the cytoplasmic surface of the mammalian erythrocyte plasma membrane and is believed to confer upon this cell membrane its properties of strength and elasticity (1, 2). Protein 4.1 facilitates the interaction of spectrin and actin by forming a ternary complex with these components and also has been shown to interact directly with two intrinsic membrane proteins, glycophorin and the anion transporter, and membrane phospholipid (2). Protein 4.1, therefore, plays a key role in the maintenance of the membrane skeleton. Protein 4.1 exists as a set of structurally related polypeptides in avian erythroid cells (3). In chicken erythrocytes, seven major protein 4.1 variants of 77, 87, 100, 115, 150, 160, and 175 kDa are expressed, with the 100- and 115-kDa polypeptides found in highest abundance (3, 4). However, in immature mitotic erythroblasts, the 77- and 87-kDa protein 4.1 variants predominate, and as the cells undergo terminal differentiation, the 100- and 115-kDa forms are found at higher levels (4, 5). Protein 4.1 and protein 4.1 analogues also have been localized in nonerythroid tissues (3, 4, 6–11).

To define the mechanism(s) by which multiple protein 4.1 variants are generated, we have isolated cDNA clones encoding chicken protein 4.1. Partial DNA sequence analysis demonstrates 86% homology with human protein 4.1 cDNA sequences (12) over a 270-base-pair portion of a chicken protein 4.1 cDNA. We find that the gene encoding protein 4.1 exists as a single copy in the haploid chicken genome and gives rise to one 6.6-kilobase (kb) size-class of RNA. By S1 nuclease mapping experiments, we demonstrate that this single size-class RNA consists of multiple, distinct mRNAs, whose expression is developmentally regulated during chicken embryonic erythropoiesis. Hence, a heterogeneity of protein 4.1 mRNAs appears to underlie the diversity of protein 4.1 polypeptides. A comparison of protein 4.1 polypeptides synthesized *in vitro* and *in vivo* shows that the relative abundance of protein 4.1 variants is further regulated at the translational level.

MATERIALS AND METHODS

Isolation of Protein 4.1 cDNAs. Protein 4.1-specific cDNAs were isolated from a λgt11 expression library constructed from 14- to 15-day chicken embryo erythroid poly(A)$^+$ RNA ("M" library of ref. 13). The library was screened with a protein 4.1-specific antiserum (3), and positive plaques were isolated and rescreened several times, as described (13). Complementary DNA inserts of candidate protein 4.1 clones were isolated from *Eco*RI-digested recombinant phage DNA and inserted into pT7SP6 (a gift of V. Axelrod, Columbia University) (14) or m13mp19. DNA sequence analysis was performed by the dideoxynucleotide chain-termination procedure (15).

RNA Preparation. Total cellular or cytoplasmic poly(A)$^+$ RNA was prepared from chicken embryonic erythroid cells as described (13). To enrich for protein 4.1 mRNA, cytoplasmic poly(A)$^+$ RNA was fractionated on a formamide/sucrose gradient, as described (13). Fractions were collected, RNA was concentrated by ethanol precipitation, and fractions containing protein 4.1 translational activity in a rabbit reticulocyte lysate (16) were pooled.

***In Vitro* Translation and Hybridization-Selected Translation.** Poly(A)$^+$ RNA was translated *in vitro* for 90 min at 30°C in a high-activity nuclease-treated rabbit reticulocyte lysate containing [^{35}S]methionine (13, 16). Complete translation reactions or immunoprecipitates using a protein 4.1 antiserum (3) were resolved on 12.5% polyacrylamide/NaDodSO$_4$ gels (17). For an *in vivo* protein 4.1 standard, 15-day chicken embryo erythroid cells were metabolically labeled with [^{35}S]methionine for 30 min at 37°C, fractionated with Triton X-100, and immunoprecipitated with the protein 4.1 antiserum, as described (3). Labeled protein bands were visualized by fluorography of gels impregnated with 2,5-diphenyloxazole (18). Plasmid DNA was bound to nitrocellulose and subjected to positive hybridization-selected translation, as described (13, 19). RNA selected from 10 μg of gradient-enriched poly(A)$^+$ RNA was translated in 15 μl of rabbit reticulocyte lysate.

DNA and RNA Analysis. DNA was digested with restriction endonucleases, electrophoresed, blotted to nitrocellulose, and protein 4.1 sequences were detected with ^{32}P-labeled nick-translated cDNA inserts (20, 21). RNA blots were as described (22, 23). S1 nuclease protection (24) of a protein 4.1

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Abbreviation: nt, nucleotide(s).

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cDNA was performed by hybridizing RNA with an end-labeled 3.1-kb *Xho* I–*Pvu* I fragment of pFPO20, as described in the legend of Fig. 5.

RESULTS

Isolation of Chicken Protein 4.1 cDNAs. We identified cDNA clones coding for protein 4.1 from a λgt11 expression library (13) by screening with an antiserum specific for chicken protein 4.1 (3). DNA sequence analysis of the 270 nucleotides (nt) at the 5′ end of one cDNA, pFPO20, reveals 86% nucleotide homology with a human reticulocyte protein 4.1 cDNA (12), rendering 98% amino acid sequence conservation from chicken to human protein 4.1 in this region (Fig. 1*a*). A restriction endonuclease map of representative protein 4.1 cDNAs is shown in Fig. 1*b*; we conclude from the above data that the cDNAs shown are specific for protein 4.1.

***In Vitro* Translation of Chicken Protein 4.1 and Positive Hybridization-Selected Translation.** To examine the mRNA(s) encoding the protein 4.1 polypeptides, we analyzed [^{35}S]methionine-labeled translation products synthesized in a rabbit reticulocyte lysate (13, 16), using cytoplasmic poly(A)$^+$ RNA isolated from circulating erythroid cells of 15-day chicken embryos. The seven major protein 4.1 variants of 77, 87, 100, 115, 160, and 175 kDa were immunoprecipitated from the *in vitro* translation mixture; the 160- and 175-kDa polypeptides were in greatest abundance (Fig. 2*a*, lane 2). However, when protein 4.1 was immunoprecipitated from cytoskeletal extracts of 15-day embryo erythroid cells metabolically labeled with [^{35}S]methionine, the 100- and 115-kDa polypeptides were the predominant forms, and the 77-, 87-, 150-, 160-, and 175-kDa variants were found in lower relative amounts (3–5) (see Fig. 2 *a*, lane 1, and *b*, lane 8). As the protein 4.1 polypeptides are rapidly stabilized in the membrane skeleton with equivalent efficiencies shortly after their synthesis (3–5), the pattern of protein 4.1 variants identified after labeling *in vivo*, shown in Fig. 2, closely approximated their ratios of synthesis as well as the steady-state ratios (3). The relative rates of synthesis of protein 4.1 variants *in vitro* and *in vivo*, therefore, differ significantly.

Protein 4.1 was a minor *in vitro* translation product and was not identifiable in the total translation reaction mixture (compare Fig. 2*a*, lanes 2 and 3). To increase the relative abundance of protein 4.1 mRNA, we fractionated 15-day embryo erythroid poly(A)$^+$ RNA on a denaturing 4–20% sucrose gradient and pooled RNA fractions with protein 4.1 translational activity. Translation of this material *in vitro* showed that this preparation was substantially depleted of globin mRNA (Fig. 2*b*, lane 1; compare with Fig. 2*a*, lane 3) and directed the translation of the protein 4.1 polypeptides (Fig. 2*b*, lane 2). The immunoprecipitated 87-, 100-, and 115-kDa variants were more visible over the reduced background from the gradient-enriched RNA translation mixture than from the unfractionated RNA translation mixture (Fig. 2*a*, lane 2). We, therefore, used gradient-enriched RNA for subsequent hybridization-selected translation experiments.

We performed hybridization-selected translations to further determine the structural relatedness of the protein 4.1 polypeptides. Fig. 2*b* shows that a representative cDNA, pFPO2a, hybridized to RNA that directed the synthesis of protein 4.1 polypeptides (lane 5). In contrast, the plasmid vector alone did not hybridize to any detectable protein 4.1 RNA (lane 4). The hybridization-selected translation products shown in lane 5 were specifically immunoprecipitated by an anti-protein 4.1 antiserum (lane 7), whereas no protein 4.1 translation products were immunoprecipitated from the plasmid vector-mediated translation reaction mixture (lane 6). Positive hybridization-selected translation was repeated using pFPO2a, pFPO2b, and pFPO20, with results similar to those shown in Fig. 2 (data not shown). We conclude that the cDNAs described here, together representing ≈3.5 kb, or 55% of protein 4.1 mRNA (see below), are complementary to the mRNA(s) encoding all the major protein 4.1 variants found in 15-day embryo erythroid RNA.

Representation of Protein 4.1 Sequences in the Chicken Genome. Blot analysis of chicken genomic DNA using ^{32}P-labeled protein 4.1 cDNA probes shows that the multiple protein 4.1 polypeptides are encoded by a single gene (Fig. 3). pFPO2a hybridized to several genomic DNA bands generated by digestion with *Bam*HI, *Hin*dIII, *Eco*RI, or *Xba* I (Fig.

a

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HUMAN:      1324 GAG TTT CTT GAG AAT GCC AAA AAG TTG TCT ATG TAT GGA GTT GAT CTT CAT AAA GCA AAG GAC TTG GAA GGA GTA GAT ATC ATC CTA GGT
CHICKEN:       1 GAG TTC CTC GAG AAT GCA AAG AAA CTG TCT ATG TAT GGA GTT GAC CTT CAC CAC GCC AAG GAC TTG GAA GGA GTG GAT ATC ACT CTG GGA
CHICKEN AA:      glu phe leu glu asn ala lys lys leu ser met tyr gly val asp leu his his ala lys asp leu glu gly val asp ile thr leu gly
                                                                                    (lys)                                (ile)

HUMAN:      144 GTC TGC TCT AGT GGC CTT CTG GTT TAC AAA GAT AAG CTG AGA ATT AAC CGC TTC CCT TGG CCC AAA GTG CTG AAG ATT TCT TAT AAA CGT
CHICKEN:     91 GTC TGT TCC AGT GGC CTT CTT GTT TAC AAA GAT AAG CTG AGA ATC AAC CGC TTC CCT TGG CCC AAA GTC CTG AAG ATT TCC TAC AAA CGC
CHICKEN AA:      val cys ser ser gly leu leu val tyr lys asp lys leu arg ile asn arg phe pro trp pro lys val leu lys ile ser tyr lys arg

HUMAN:     1504 AGT AGC TTT TTC ATC AAG ATT CGG CCT GGA GAG CAA GAG CAG TAT GAA AGT ACC ATC GGA TTC AAA CTT CCC AGT TAC CGA GCA GCT AAG
CHICKEN:    181 AGC AGC TTC TTT ATT AAG ATT CGC CCA GGG GAG CAA GAG CAG TAC GAA AGT ACA ATT GGA TTC AAG CTA CCA AGT TAC CGG GCA GCA AAG
CHICKEN AA:      ser ser phe phe ile lys ile arg pro gly glu gln glu gln tyr glu ser thr ile gly phe lys leu pro ser tyr arg ala ala lys
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b

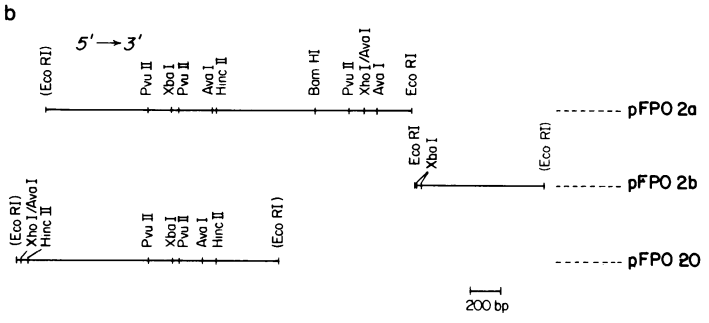

FIG. 1. Protein 4.1 cDNAs. (*a*) DNA sequence analysis of the 270 nt at the 5′ end of pFPO20. Top row: sequence of a human protein 4.1 cDNA from nt 1324 to nt 1593, according to Conboy *et al.* (12). Middle row: sequence of pFPO20. Dots indicate nonhomologous nucleotides. Bottom row: Amino acid sequence derived from the chicken protein 4.1 cDNA. Nonhomologous human protein 4.1 residues are indicated in parentheses. (*b*) Restriction endonuclease map of protein 4.1 cDNAs. pFPO2a and pFPO2b were isolated from the same λgt11 recombinant (λFPO-2). *Eco*RI sites in parentheses are from synthetic linkers, whereas the *Eco*RI site shared by pFPO2a and pFPO2b occurs naturally. The direction of transcription of pFPO2a and pFPO2b was determined by their orientation within the β-galactosidase gene of λgt11 (25). pFPO20 was isolated subsequent to back-screening of the λ cDNA library with the pFPO2a insert.

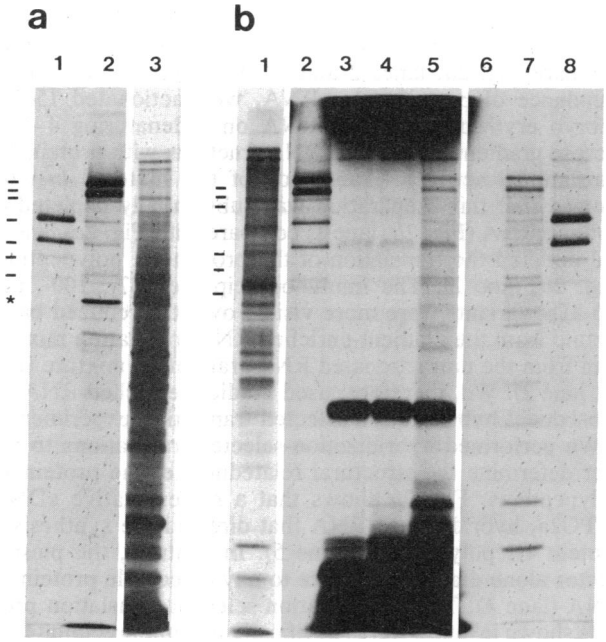

FIG. 2. Synthesis of protein 4.1 *in vivo* and *in vitro* and identification of protein 4.1-specific cDNAs. (*a*) $NaDodSO_4$/polyacrylamide gel electrophoretic analysis of protein 4.1 synthesis. Lanes: 1, immunoprecipitate of protein 4.1 from cytoskeletal extracts of 15-day embryo erythroid cells labeled *in vivo*; 2, immunoprecipitate from 10 μl of rabbit reticulocyte lysate in which 15-day embryo erythroid cytoplasmic poly(A)$^+$ RNA was translated; 3, 1 μl of the complete *in vitro* translation reaction mixture used in lane 2. The three high molecular weight bands in lane 3 are ankyrin, α-spectrin, and β-spectrin, in descending order. Bars indicate positions of protein 4.1 variants (from top to bottom): 175 kDa, 160 kDa, 150 kDa, 115 kDa, 100 kDa, 87 kDa, and 77 kDa. The asterisk marks the position of the 70-kDa heat shock protein, which is a major translation product of 15-day embryo erythroid RNA and which is immunoprecipitated nonspecifically from the *in vitro* translation reaction mixtures. Lanes 1 and 2 were fluorographed for 8 days, whereas lane 3 was exposed for 16 hr. (*b*) $NaDodSO_4$/polyacrylamide gel analysis of gradient-enriched erythroid poly(A)$^+$ RNA translation products and hybridization-selected translation products. Lanes: 1, 0.5 μl of an *in vitro* translation of gradient-enriched 15-day embryo erythroid poly(A)$^+$ RNA; 2, immunoprecipitate from 10 μl of the *in vitro* translation reaction mixture shown in lane 1; 3, endogenous translational activity from 5 μl of lysate with no added RNA; 4, products of hybridization-selected translation using plasmid vector (5 μl of a 15-μl reaction mixture); 5, products of hybridization-selected translation mediated by pFPO2a (5 μl); 6 and 7, protein 4.1-specific immunoprecipitate from 10 μl of lysate shown in lanes 4 and 5, respectively; 8, immunoprecipitate of metabolically-labeled protein 4.1, as in *a*, lane 1. Bars indicate positions of protein 4.1 polypeptides, as in *a*. Lanes 1–8 represent consecutive lanes from the same gel, but were fluorographed for 1 day (lane 1), 3 days (lanes 2–5), or 6 days (lanes 6–8).

3*a*). When the 1.7-kb *Eco*RI–*Bam*HI 5′ end fragment or the 0.7-kb *Bam*HI–*Eco*RI 3′ end fragment of pFPO2a was used as probe (see Fig. 1*a*), each hybridized to a subset of the genomic bands obtained with the entire cDNA insert (Fig. 3 *b* and *c*). A similar hybridization using pFPO2b as probe identified the same *Bam*HI, *Hin*dIII, and *Xba* I genomic fragments detected by the pFPO2a 3′ probe (Fig. 3 *c* and *d*, lanes 1–3), but since pFPO2a and pFPO2b share a common terminal *Eco*RI site, pFPO2b hybridized to a genomic *Eco*RI fragment that was not detected by pFPO2a (Fig. 3 *a–d*, lanes 4). The hybridization patterns shown in Fig. 3 *a–d*, in conjunction with a preliminary analysis of overlapping protein 4.1 genomic DNA phage recombinants (unpublished observations), indicate that the multiple bands detected in protein 4.1 genomic DNA blots are contiguous; pFPO2a and pFPO2b hybridize to overlapping cloned genomic sequences

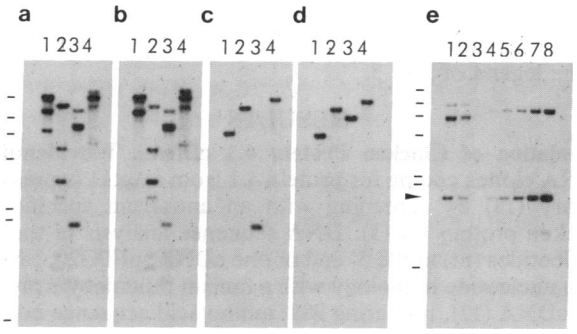

FIG. 3. Blot analysis of chicken genomic DNA using protein 4.1 cDNA probes. (*a–d*) One microgram of chicken liver DNA was digested with *Bam*HI (lane 1), *Hin*dIII (lane 2), *Eco*RI (Lane 3), or *Xba* I (lane 4), electrophoresed on a 0.9% agarose gel, and blotted to nitrocellulose. Blots were hybridized to the following ^{32}P-labeled nick-translated cDNA sequences. (*a*) pFPO2a insert. (*b*) The 1.7-kb pFPO2a 5′ *Eco*RI–*Bam*HI fragment. (*c*) The 0.7-kb pFPO2a 3′ *Bam*HI–*Eco*RI fragment. (*d*) pFPO2b insert. The autoradiograms in *a–d* were from adjacent strips of nitrocellulose blotted to the same gel. The positions of λ DNA digested with *Hin*dIII are shown (23, 9.4, 6.6, 2.3, 2.0, and 0.56 kb). (*e*) Determination of protein 4.1 gene copy number. A recombinant phage, λ4.1-19, containing chicken protein 4.1 gene sequences was isolated from a λ Charon 4A genomic library (26) and used to quantitate protein 4.1 DNA hybridization. Chicken liver DNA (1.0 μg and 0.5 μg) and λ 4.1-19 DNA were digested with *Eco*RI, separated on a 0.9% agarose gel, blotted to nitrocellulose, and hybridized to ^{32}P-labeled pFPO2a. The 1.8-kb *Eco*RI band (arrowhead) was used to calibrate the hybridization signals. Lanes: 1, 1.0 μg of chicken liver DNA; 2, 0.5 μg of chicken liver DNA; 3–8, λ4.1-19 DNA corresponding to 0.25, 0.5, 1.0, 2.0, and 4.0 copies per haploid genome (C value = 1.5 pg) per μg of chicken DNA, respectively. Bars denote λ *Hin*dIII-digested DNA size markers.

spanning over 40 kb (unpublished observations). To determine the copy number of the protein 4.1 gene(s) in the chicken genome, known amounts of genomic DNA or DNA of a protein 4.1 genomic clone, λ4.1-19, were digested with *Eco*RI, and the relative hybridization of the 1.8-kb *Eco*RI fragment (an internal restriction fragment in the λ4.1-19 insert; data not shown) with ^{32}P-labeled pFPO2a was used to determine gene copy number (see Fig. 3 legend). The autoradiogram of Fig. 3*e* shows that the 1.8-kb *Eco*RI band detected (arrowhead) was found at one copy per haploid genome (compare lanes 1 and 6 or lanes 2 and 5). Since pFPO2a is complementary to RNA(s) encoding all the major protein 4.1 polypeptides (Fig. 2*b*, lanes 5 and 7), we conclude that multiple protein 4.1 variants arise from a single gene.

Multiple Protein 4.1 mRNAs of Indistinguishable Sizes Encode Multiple Chicken Protein 4.1 Polypeptides. The experiments shown in Figs. 2 and 3 suggest that multiple protein 4.1 variants arise by translationally regulated expression of one or several mRNA(s) encoded by a single gene. RNA blot analysis of gradient-enriched 15-day embryo erythroid poly(A)$^+$ RNA using ^{32}P-labeled pFPO2a as probe revealed a single major 6.6-kb RNA (Fig. 4*b*, lane I). Several minor bands smaller than the 6.6-kb RNA were also detected amid a trail of degradation (Fig. 4*b*, lane I), in a pattern similar to that found in unfractionated poly(A)$^+$ RNA (data not shown). To determine if these minor species were protein 4.1 mRNAs or stable degradation intermediates, we separated the gradient-enriched RNA on a preparative methylmercury hydroxide agarose gel and analyzed gel-fractionated RNA by *in vitro* translation or by RNA blot analysis (Fig. 4). The autoradiograms of Fig. 4 *a* and *b* show that translation of each major protein 4.1 variant cofractionates in gel slices 4–7 (Fig. 4*a*), which corresponds to the major 6.6-kb protein 4.1 RNA peak (Fig. 4*b*). Within this peak, the ratios of the protein 4.1 polypeptides remained constant from fraction to fraction. The resolution of this experiment was sufficient to separate

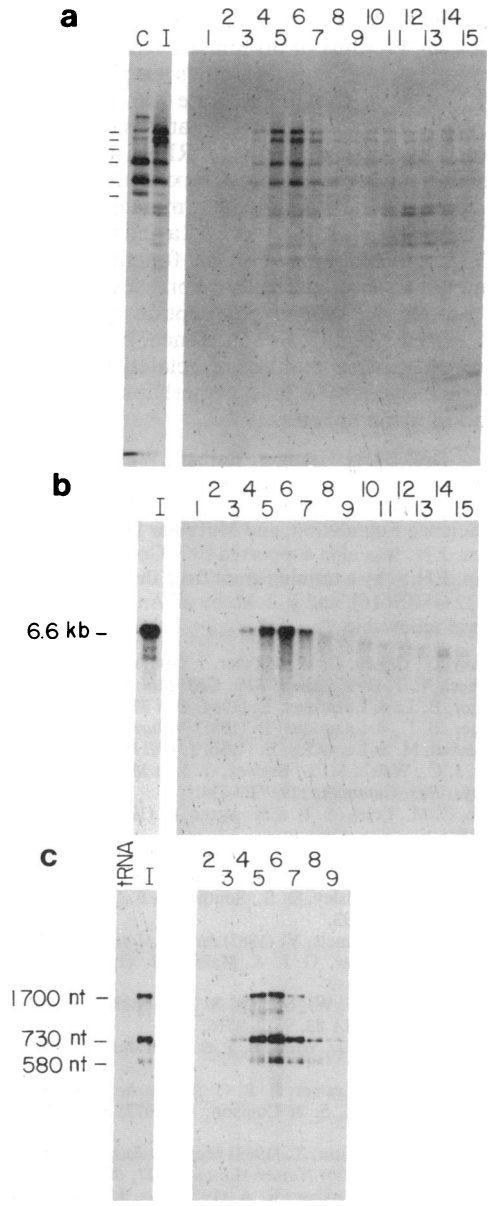

FIG. 4. Fractionation of 15-day embryo erythroid RNA on a methylmercury hydroxide agarose gel and analysis by *in vitro* translation, RNA blotting, and S1 nuclease protection. Gradient-enriched poly(A)$^+$ RNA was electrophoresed in the presence of methylmercury hydroxide (27) on a preparative 0.9% low gelling temperature agarose gel. The gel was sliced in 0.5-mm-thick sections perpendicular to the axis of electrophoresis, and RNA was isolated as described (22). (*a*) *In vitro* translation of fractionated RNA. RNA was translated in a rabbit reticulocyte lysate containing [^{35}S]methionine, immunoprecipitated with protein 4.1 antiserum, and analyzed on a 12.5% polyacrylamide/NaDodSO$_4$ gel. Lanes: C, immunoprecipitate of protein 4.1 from 15-day embryo erythroid cells labeled with [^{35}S]methionine; I, immunoprecipitation from reticulocyte lysate in which gradient-enriched poly(A)$^+$ RNA ("input") was translated; 1–15, immunoprecipitates from translation reaction mixtures of fractionated RNA from gel slices 1–15, respectively. Bars indicate 175, 160, 150, 115, 100, and 87 kDa protein 4.1 variants. (*b*) RNA blot of fractionated RNA. RNA was electrophoresed in the presence of formaldehyde, blotted to nitrocellulose, and probed with ^{32}P-labeled pFPO2a cDNA insert. Lanes: I, gradient-enriched RNA ("input"); 1–15, RNA from slices 1–15. (*c*) S1 nuclease analysis of fractionated RNA. S1 nuclease mapping was performed using a ^{32}P-end-labeled pFPO20 cDNA probe (see Fig. 5 legend). Protected fragments were separated on a 1.5-mm-thick 7 M urea/5% polyacrylamide gel. tRNA, ^{32}P-labeled DNA protected after incubation with tRNA. Lane I, fragments protected by gradient-enriched poly(A)$^+$ RNA ("input"). Lanes 2–9, fragments protected by RNA in slices 2–9.

the streak of degraded protein 4.1 RNA, as well as the minor RNA species, from the protein 4.1 mRNA peak in slices 4–7. Hence, multiple protein 4.1 polypeptides are derived from a single mRNA size-class.

Although a single size of mRNA appeared to give rise to multiple protein 4.1 products, we could not rule out the existence of multiple protein 4.1 mRNAs with indistinguishable electrophoretic mobilities. We, therefore, performed S1 nuclease digestions on hybrids formed between erythroid protein 4.1 RNA and an end-labeled protein 4.1 cDNA. For this purpose, a probe was prepared that consisted of a pFPO20 fragment end-labeled at the *Xho* I site (see Fig. 1*a*). Fig. 5, lane 2, shows that the pFPO20 probe was protected by four distinct RNA species from erythroid cells of 15-day chicken embryos. The 1700-nt protected fragment identifies an RNA species complementary to pFPO20 from the *Xho* I site to the 3′ terminus of the insert. Additional fragments are present at 730 nt, 580 nt, and 470 nt (barely visible in this exposure; see below) and represent RNAs that are derived from DNA containing this *Xho* I site, but are divergent 730, 580, and 470 nt downstream. S1 nuclease analysis of RNA fractionated on the same methylmercury hydroxide agarose gel described in Fig. 4 demonstrates that the multiple protein 4.1 RNAs coelectrophorese at 6.6 kb (Fig. 4*c*), precluding the possibility that the multiplicity of protected fragments arose from RNA precursors or degradation products. A greater heterogeneity of protein 4.1 mRNA may well exist, as other putative RNAs that do not contain sequences both complementary to and continuous with the *Xho* I site within this 1.7-kb cDNA would not be detected.

Expression of Multiple Protein 4.1 mRNAs Is Developmentally Regulated. Circulating erythroid cells of 4-day embryos are "early" mitotic erythroblasts of the primitive series,

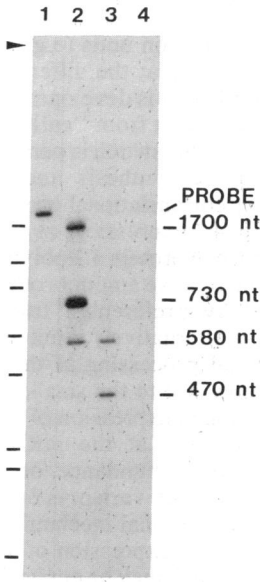

FIG. 5. S1 nuclease protection of protein 4.1 cDNA by chicken erythroid RNA. pFPO20 was cleaved with *Xho* I and end-labeled by filling out with DNA polymerase I large fragment in the presence of [α-^{32}P]dNTPs (see Fig. 1*b*), digested with *Pvu* I, and the 3.1-kb fragment containing 1.7-kb insert plus 1.4-kb vector sequences was isolated and hybridized with RNA in 80% (vol/vol) formamide, 40 mM Pipes (pH 6.4), 0.4 M NaCl, 1 mM EDTA at 50°C for 15 hr. Samples were digested with S1 nuclease (24), and fragments were resolved on 0.3-mm-thick denaturing 7 M urea/5% polyacrylamide gels. Lanes: 1, undigested probe; 2, fragments protected by 2 μg of cytoplasmic poly(A)$^+$ RNA of 15-day embryo erythroid cells; 3, fragments protected by 2 μg of total cellular poly(A)$^+$ RNA of 4-day embryo erythroid cells; 4, S1 nuclease digestion following hybridization with 2 μg of tRNA. Bars indicate mobilities of end-labeled DNA markers (1856, 1060, 929, 622, 527, 404, 383, and 309 nt).

whereas circulating erythroid cells of 15-day embryos are "late" postmitotic definitive series cells (36). The pattern of protein 4.1 variant expression in embryonic erythroid cells changes during ontogeny as well as within the primitive series and definitive series lineages (4). To compare a "late" pattern of protein 4.1 mRNA expression (Fig. 5, lane 2) with a pattern from "early" cells, we performed S1 nuclease analysis on RNA from 4-day embryos. Using the ^{32}P-end-labeled probe described above, we found protection of only the 470-nt and 580-nt fragments by the 4-day embryo erythroid RNA (Fig. 5, lane 3). Blot analysis of this RNA detects a single 6.6-kb protein 4.1-specific band (data not shown). We interpret the results described above to indicate that the generation of multiple protein 4.1 mRNAs is developmentally regulated.

DISCUSSION

We have examined the mechanisms by which multiple chicken protein 4.1 polypeptides are generated, using cloned cDNA probes specific for protein 4.1. Our protein 4.1 cDNAs are complementary to RNAs encoding all the major chicken erythroid protein 4.1 polypeptides, as shown by hybridization-selected translations. Quantitative genomic DNA blotting reveals that multiple protein 4.1 variants arise from a single gene. By RNA blot analysis and S1 nuclease mapping, we have determined that the single protein 4.1 gene generates multiple mRNAs with indistinguishable sizes. The observation of multiple 6.6-kb mRNAs exhibiting sequence discontinuities at distances of up to ≈1 kb apart indicates that alternative pathways of pre-mRNA splicing (e.g., see refs. 28–30) play a key, but not necessarily exclusive, role in the genesis of multiple protein 4.1 mRNAs. Different sites of transcription initiation as well as termination and/or transcript cleavage and polyadenylylation may also occur and indeed may determine the selection of splicing patterns, as shown for other transcription units (e.g., see refs. 31–33).

In this study we show that the differential expression of multiple protein 4.1 mRNAs is developmentally regulated, as the mRNAs detected switch from "early" primitive cells to "late" definitive cells. This switch is paralleled by changes in protein 4.1 polypeptide synthesis and accumulation (4). Translational and/or cotranslational processes also regulate the pattern of protein 4.1 expression, as demonstrated by the quantitative difference in protein 4.1 polypeptide synthesis *in vivo* and *in vitro*. The relative amounts of protein 4.1 variants may be controlled by preferential translation of certain protein 4.1 mRNAs. Alternatively, limited cotranslational or rapid posttranslational processing of the larger protein 4.1 polypeptides may give rise to the smaller variants (see refs. 3 and 5). Hence, although expression of protein 4.1 polypeptides is specified initially at the mRNA level by RNA processing, the relative abundance or perhaps even the presence or absence of each variant is further determined by translational or cotranslational mechanisms.

Our data regarding the expression of multiple protein 4.1 mRNAs from a single gene may be applicable to the observed occurrence of protein 4.1 and protein 4.1 analogues in nonerythroid tissues (3, 4, 6–9, 11). For example, in developing chicken lens, protein 4.1 variants similar to those found in chicken erythroid cells are present (3, 4). A 6.6-kb protein 4.1 mRNA is detected in chicken lens by RNA blotting (data not shown), suggesting that lens protein 4.1 is specified by the same gene that encodes erythroid protein 4.1. The significance of producing multiple protein 4.1 mRNAs and polypeptides in a developmentally regulated and tissue-specific manner remains to be determined. It is possible that differential splicing of protein 4.1 exons facilitates the expression of alternative functional domains, which would affect or mediate the interactions of protein 4.1 with other membrane skeletal components. Future studies employing full-length protein 4.1 cDNAs should facilitate our understanding of both the structural and regulatory features of the differential expression of multiple protein 4.1 mRNAs and polypeptides.

Mammalian erythroid protein 4.1 exists as two structurally related polypeptides with molecular masses of 80 kDa (4.1a) and 78 kDa (4.1b) (34, 35); protein 4.1a and 4.1b differ at their carboxyl termini but appear to be functionally equivalent (35). Mammalian lens generally expresses a minor 125- to 145-kDa protein 4.1-related polypeptide in addition to the protein 4.1a/b doublet (3, 6, 11). Hence, mammalian protein 4.1 is expressed as a number of related variants, but the extent of heterogeneity is less, or perhaps more subtle, than that found in avian species.

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